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EVALUATION OF OVOSTATIN AND OVOSTATIN ASSAY

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INTRODUCTION

Ovostatin is a 780,000 MW protein, originally isolated from chicken egg white, which is active as a protease inhibitor (1). Structural studies indicate that the protein is a tetramer of identical subunits of 165,000 MW which can be separated upon reduction with β -mercaptoethanol. Chicken ovostatin is an inhibitor of metalloproteases such as collagenase and thermolysin, and of acid proteases such as pepsin and rennin (2). Ovostatin isolated from duck eggs (3) and from crocodile eggs (4) appears to be similar to chicken egg ovostatin, but with significant differences in structure and function. Duck ovostatin contains a reactive thiol ester which is not found in the chicken protein, and duck and crocodile ovostatin inhibit serine proteases such as trypsin and chymotrypsin, while chicken ovostatin does not. Electron microscopy (4,5) of ovostatin indicates that two subunits associate near the middle of each polypeptide to form a dimer with four arms. Two of these dimers then associate to produce a tetramer with eight arms, with the protease binding site near the center of the molecule. Upon binding of the protease, a conformational change causes all eight arms to curl toward the center of the molecule, effectively trapping the protease and sterically hindering access of the substrates to its active site. The structural organization and mechanism of action proposed for ovostatin are nearly identical to that proposed for α_2 -macroglobulin, a serum protease inhibitor (6) which may play an important role in regulation of proteases in animal tissues.

Although the general arrangement of subunits appears to be the same for all ovostatins studied, some differences have been observed, with chicken ovostatin more closely resembling reptilian ovostatin than the duck protein. This is a surprising result, given the evolutionary relatedness of chickens and ducks. It is possible that the difference in structures may be due to deformed subunit arrangements which occur during the processing and fixing necessary for electron microscopy (4). Examination of the native structure of these proteins using X-ray crystallography would help clarify these discrepancies.

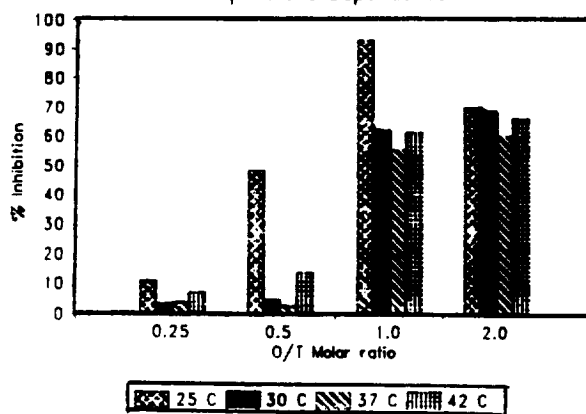
BODY

Obviously, it is necessary to have good quality crystals of ovostatin if x-ray crystallography is to be performed. Such crystals could also be used as a model system to study and understand numerous aspects of crystal growth for such a large protein. For these reasons, attempts have been made at MSFC to prepare crystals of chicken egg white ovostatin. Ovostatin has been purified using slight modifications of published procedures. SDS-gel electrophoresis under reducing conditions

indicated a large band of MW 165,000 and a smaller band at MW 88,000. This smaller band has been reported to be a fragment produced by action of the bound protease on the ovostatin (7) and has also been found to occur due to autolytic degradation of duck ovostatin. Such autolytic degradation had not previously been observed for chicken ovostatin (7). Attempts to crystallize the ovostatin preparations have had limited success, with reasonable size crystals only occurring on a few occasions. For this reason, it was deemed necessary to investigate the protease inhibitory activity of the ovostatin preparations to determine if native, active molecules were in fact being purified.

One assay for ovostatin employs the metalloprotease thermolysin and uses azocasein as its substrate in a reaction carried out at 23°C. Nagase et al. (1) have reported that using this assay, they have observed a 1:1 stoichiometric relationship between thermolysin and ovostatin. Thus, when there is a molar ratio of ovostatin:thermolysin of 0.5 one should observe 50% inhibition of the protease. Initial trials using this assay at MSFC resulted in absorbance differences between the blanks and the positive controls of only 0.3 - 0.6 absorbance units. Also, the azocasein substrate gave higher readings with increasing storage time at 4°C. Hemoglobin was tried as an alternate substrate for the thermolysin, but was not a good substrate for the enzyme. After several preparations of new azocasein solutions it was found that storing the azocasein solution at -20°C gave more stable, low blank values for the assay. Increasing the assay temperature from 23°C to 37°C increased the activity of the thermolysin and hence, the absorbance readings, as expected. However, it was observed that ovostatin inhibition of thermolysin was decreased at molar ratios of ovostatin:thermolysin less than 1.0. The observed temperature dependence of the assay is shown in Figure 1. Since ovostatin is expected to be a physiologically important inhibitor of bacterial proteases in the egg at the normal chicken body temperature of 42° C these results are curious and warrant further investigation.

Figure 1. Ovostatin Inhibition of Thermolysin
Temperature Dependence



Several ovostatin preparations were assayed and found to yield less than a 50% inhibition of the thermolysin when used at a 0.5 molar ratio of ovostatin:thermolysin. These preparations were analyzed by SDS-polyacrylamide electrophoresis, and all but one appeared to be quite pure, except that the 88K MW degradation product was visible in nearly all the lyophilized, stored preparations. Assay and gel electrophoresis were then performed on freshly prepared ovostatin at several key steps during the purification procedure. The preparation did not have much of the 88K band present and seemed to be nominally active through the ion exchange column portion of the isolation procedure. At this point it was also observed that the ovostatin solutions stored at 4°C appeared to lose activity with time. Thus, preparations of ovostatin that required more than 5-6 days to complete could be becoming less active during the isolation.

Many of the blood coagulation factors are proteases and it was of interest to determine whether ovostatin might inhibit one or more of these. Thrombin, which acts near the end of the blood clotting cascade, is readily available commercially, so ovostatin was examined for its ability to inhibit the action of thrombin on fibrinogen and the subsequent formation of a fibrin clot. Assays at 37°C with up to a 2 fold molar excess of ovostatin over thrombin did not indicate any inhibition. Native polyacrylamide gels of ovostatin incubated with thermolysin or with thrombin indicated that the thermolysin bound to ovostatin and changed its electrophoretic mobility, but the thrombin did not.

Assays of ovostatin performed at both high (1.0 mg/ml) and low (0.025 mg/ml) concentrations gave conflicting and irreproducible results. It was thought that perhaps there was either an as yet unreported requirement for some cation for ovostatin activity, or that some cation could inactivate the ovostatin. To test this hypothesis, ovostatin was incubated with 1 mM EDTA prior to incubating it with thermolysin. The results of this experiment indicated that this treatment may have produced a slight increase in the activity of the ovostatin when assayed at a molar ratio of 0.5. However, incubation of ovostatin with 5 mM EDTA resulted in the opposite effect, decreasing the ovostatin activity at a 0.5 molar ratio.

Several attempts were made to crystallize different ovostatin preparations that had been stored lyophilized at -20°C, but none were successful.

CONCLUSIONS

As is often the case in science, these results have

raised more questions than they have answered. While it appears that ovostatin prepared at MSFC has some inhibitory activity towards thermolysin, it may not have optimal activity. This may or may not be the reason for the difficulty in crystallizing these preparations. Although the crystallization problem was not solved, several important observations were made:

- 1) Azocasein solutions must be stored at -20° C.
- 2) Thermolysin solutions should be made up as concentrated solutions in 50% glycerol, stored at -20° C and diluted to the appropriate concentration immediately before use.
- 3) Hemoglobin is not a good substrate for this assay.
- 4) Chicken ovostatin does not inhibit thrombin.
- 5) The inhibition of thermolysin by ovostatin is temperature dependent at low ovostatin:thermolysin ratios, and decreases as one approaches physiological temperatures.
- 6) It appears that there are as yet undefined variables in the purification of active chicken ovostatin.

More work needs to be done to identify the reason for the appearance of the 88K MW band in the ovostatin preparations and to discern the appropriate conditions to produce ovostatin crystals.

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